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TECHNIQUES FOR STUDYING THE EFFECTS OF MICROGRAVITY ON MODEL PARTICLE/CELL SYSTEMS

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ABSTRACT

In an effort to learn more about the effects of a simulated low gravity environment on skeletal muscle, skeletal muscle cell cultures were grown within the lumen of XM-80 hollow fibers (i.d. = 0.5 mm) in a Clinostat rotating at 100 rpm. Cells were isolated from the thigh muscle tissue of 12 day embryos and were cultured for up to 14 days in the hollow fiber environment. Cells proliferated to confluency within several days, and fusion into multinucleated myotubes was then apparent. Fibers were stretched by a built-in spring mechanism to hold the fiber tightly at the center of rotation, and sections of the fiber were removed at 3, 7 and 14 days for electron microscopic analysis. When the Clinostat is rotated in the horizontal position, the gravity vector approaches zero and the cells are in an environment that simulates microgravity. Control experiments consist of one fiber rotated in the vertical position in the clinostat and another fiber that is held in a horizontal configuration in a comparable sized tube that is not rotated at all. Examination of skeletal muscle cells by electron microscopy revealed that myoblast fusion and myofibril accumulation were extensive. Two general conclusions were apparent from this investigation. First, muscle cells undergo the normal progression of proliferation, fusion and myofibril assembly in the presence of simulated microgravity for the first week in culture. After 14 days, however, many muscle fibers undergo degeneration such that myofibrillar structures are not extensive or well organized. Second, although no major abnormalities in myofibril assembly were detected in Clinostat-rotated cultures in comparison to controls that were not rotated in a Clinostat, the myofibrils in non-rotated controls tend to be more highly organized than those in either horizontally or vertically rotated Clinostat samples.

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BACKGROUND

The primary reasons for our initial interest in this research emanate from our experience in studying protein synthesis, degradation and gene expression in skeletal muscle cells, and from the extensive physical problems resulting from atrophy and weakness of skeletal and cardiac muscles following prolonged exposure to a low gravity environment (c.f., Morey-Holton and Wronski, 1981: Oganov et al., 1982; Grigor'yeva and Kozlovskaya, 1983; Leonard et al., 1983). Clearly, dramatic alterations in the balance between the rate of protein synthesis and the rate of protein degradation must accompany major changes in the quantity of muscle tissue, and it also seems exceedingly unlikely that loss of up to 25% of skeletal muscle mass could take place in the absence of switches in myofibrillar protein gene expression. The extent of skeletal muscle loss in humans and experimental animals after space flight is rather dramatic. To cite two specific examples, crew members of the first two Skylab missions maintained a negative nitrogen balance of approximately 4.5 g/day at the same time that total body potassium was also decreasing (Whedon et al., 1977). Much of this increased protein catabolism was due specifically to muscle protein degradation as evidenced by elevated rates of 3-methylhistidine excretion (Leach et al., 1979). Also, a pronounced decrease in mass and in myofibrillar cross sectional area of the soleus muscle in rats after exposure to a low gravity environment for 7 days has been reported (Goldspink et al., 1980; Riley et al., 1987).

An interesting and intriguing explanation for the effect of prolonged space flight on muscle atrophy is that a secretory defect for growth hormone (GH) may be occurring in pituitary cells (Grindeland et al., 1987a; Motter et al., 1987). Specifically, anterior pituitary cells isolated from rats flown for 7 days on the SL-3 mission secreted approximately half as much of a biologically active form of GH into culture media as ground-based controls. This observation has been qualitatively confirmed and extended by several independent approaches, including the finding that the serum concentration of GH is reduced by 50% in rats exposed to simulations of microgravity by hindlimb suspension (Motter et al., 1987).

Additionally, Grindeland et al. (1987b) have concluded that muscles of rats exposed to microgravity are significantly more resistant to exogenous and circulating GH than the skeletal muscles of control rats, since administration of GH did not alleviate muscle atrophy in animals in which it was known that GH secretion rate and serum levels of GH were also decreased by 50%. These results imply that the defect in GH utilization may extend to skeletal muscles as well.

Thus, it seems clear that exogenous factors are responsible for at least a portion of muscle atrophy; however, it also seems possible that microgravity has direct intrinsic effects on the cytoskeletal and myofibrillar contractile systems. Examples of direct effects of microgravity on cells are rather limited, but available circumstantial data are consistent with the explanation that the cytoskeletal system is involved. For example, secretory processes in general, and by definition the secretion of hormones from the pituitary, are microfilament-dependent, and it is plausible that the reduced secretion of GH described above results from a direct effect on the cytoskeletal system. Moreover, if the defect in GH utilization extends to skeletal muscle cells, and if the Insulin-like Growth Factors (IGF's, which are regulated by GH and act directly on skeletal muscle) and their receptors are internalized by cytoskeletal-dependent processes into the cytoplasm of muscle cells, then this process may also be directly affected by microgravity. Since some of the contractile proteins in the highly organized myofibrillar protein arrays in sarcomeres are nothing more than different isoforms of some of the cytoskeletal contractile proteins, an effect on expression of one class of these proteins could logically be expected to have an effect on expression of the other. Further substantiation of this possibility results from the fact that the myosin heavy chain genes are members of a rather large multigene family that may have up to twenty members in some species such as chickens, and which is known to exhibit a significant level of plasticity in its ability to have different isoforms expressed under different tissue, developmental and environmental conditions. Most crucial, however, is the fact that neither the effect of actual microgravity nor the effect of simulated microgravity on the organization, synthesis/degradation or gene expression of the contractile proteins has ever been evaluated under critically controlled conditions. One of the only possible ways to evaluate this possibility in the absence of prolonged space flights is with cultured muscle cells grown within the fibers of a rotating Clinostat. As discussed below, this instrument has the effect of mimicing a low gravity environment, and therefore allows some of the above processes to be evaluated.

OBJECTIVES

The general objective of this research was to assess the effects of exposure to simulated microgravity on ultrastructural aspects of the contractile system in chicken skeletal muscle cells using a rotating Clinostat . This general objective had two specific experimental components: (1) The progression of changes in cell morphology, fusion, and patterns of contractile filament organization in muscle cell cultures grown in hollow fibers in the Clinostat were evaluated, with appropriate controls. (2) Since it was determined that muscle cells in culture obtain their maximum amount of myofibrillar proteins after approximately one week in culture, a more extensive comparison of the myofibrillar organization in horizontally and vertically rotated cultures was carried out on the 7-day muscle cells in the hollow fibers. The primary technique for this work was electron microscopy.

METHODS AND PROCEDURES

1. Chick skeletal muscle cell cultures

Thigh muscle from 12 day broiler chick embryos was removed and disaggregated into individual cells by vortexing the muscle in growth medium on a vortex mixer at maximum speed for 20-30 seconds (Young et al., 1981). The suspension was then filtered through nylon mesh to remove connective tissue and bone, and the cells were recovered by centrifugation. Following resuspension in an appropriate amount of growth medium (Eagle's Minimum Essential Medium containing 5% chick embryo extract, 10% horse serum, 50 units/ml penicillin, 50 ug/ml streptomycin, 2.5 ug/ml fungizone) to give a concentration of 1.5×10^{10} cells/ml, the cell suspension was injected into a 70 mm long piece of 0.5 mm (inner diameter) XM-80 hollow fiber using a 1cc syringe and a 26 3/8 gauge needle. Both ends of the fiber were sealed with hot wax, and the fiber was loaded into a glass tube containing 5 ml of complete media. The fiber was held taut by a spring so that it would always be at the center of rotation of the Clinostat, and the glass tube was then sealed and the entire assembly loaded into the Clinostat.

Muscle cell cultures prepared as described above proliferate, fuse and begin to synthesize myofibrillar protein within 2-3 days in culture, and under most experimental conditions we have employed so far, they attain a maximum and constant quantity of myofibrillar proteins by approximately 7 days. Because the synthesis rate and the degradation rate must be exactly equal to each other in order to maintain a constant quantity of protein at steady-state, and because perturbations in either synthesis or degradation rates will result in a net change in the quantity and/or organization pattern of myofibrillar proteins, these cells will provide an excellent model for studying the dynamics of muscle protein accumulation and loss.

2. Microscopic Evaluation of Cells Grown in the Clinostat

A. Light Microscopy

Fibers were removed from Clinostat cultures and fixed in a 5% neutral buffered formalin solution for a minimum of 24 hours. The fibers were dehydrated through a graded ethanol series of 70%, 95%

and absolute ethanol, followed by a final treatment in xylene. The fibers were soaked in hot paraffin for 4-8 hours, embedded in the paraffin in plastic molds and sectioned with a microtome. The thin sections were rehydrated through a reverse graded alcohol series (100%, 95%, 70%) and deionized water, and subsequently stained with a hematoxylin/eosin or PAS myofibril stain using standard staining procedures. Slides were viewed and photomicrographs made at 45X and 100X. These light micrographs were useful for routine monitoring of the rate of cell growth and differentiation under the different experimental treatments.

B. Transmission Electron Microscopy

Fibers containing the cell cultures from the Clinostat were fixed in 4% buffered glutaraldehyde, followed by 1% osmium tetroxide fixation. The fibers were partially dehydrated in ethanol (25% and 50%) and stained with 1% uranyl acetate. After complete dehydration through a graded ethanol series (85%, 95% and absolute) the fibers were embedded in Spurr embedding medium and thin sectioned using a microtome. Sections were viewed and photographed with a Philips Model 201C transmission electron microscope. These electron micrographs were analyzed to determine if simulated microgravity had direct ultrastructural effects on the sarcomeric and/or cytoskeletal system in muscle cells.

To facilitate unbiased comparison of electron micrographs, samples were analyzed in a double blind fashion. That is, the samples were coded in the cell culture laboratory by someone other than the investigator doing the microscopy. Thus, the microscopist had no knowledge about the identity of the samples being sectioned and photographed. The microscopist was given instructions only to study each sample and then to take four electron micrographs from each sample that best represented that sample. Particular attention was directed to photographing representative areas containing myofibrillar structures or any unusual intracellular components. The electron micrographs shown in Figures 1-10 in this report were selected from several hundred photographs to represent consensus patterns that were observed. It should be emphasized that enormous variations in muscle samples always are present, and it is not possible to quantitate or to statistically evaluate most data that are obtained by electron microscopy.

RESULTS

Theory of simulating microgravity using a Clinostat. The fast rotating Clinostat used for these experiments was designed at MSFC and generously loaned to us by Dr. Robert Snyder. Briefly, the Clinostat is made up of a culture chamber which rotates about a horizontal axis, and an XM-80 hollow fiber containing cells is mounted in the center of rotation. Depending on the density difference between the particles and the liquid in which they are suspended, the particles may settle within circular trails. At high enough speed of rotation, cells become motionless with respect to the gravity vector and microgravity can be simulated (Brieglieb, 1983). The simulation approaches 100% for particles in suspension where centrifugal forces and Brownian motion offset each other. In the hollow fibers containing muscle cells, however, the diameter of the fiber is small enough that the centrifugal force is only approximately 0.006 x g at 100 rpm of rotation. Operation of the Clinostat in the horizontal position simulates microgravity, and operation in the vertical position serves as a control since the gravity vector is always constant in the vertical position. Additional control experiments consisting of cells in hollow fibers lying horizontally in a sealed tube of the same approximate dimensions as the chamber of the Clinostat are also conducted to ensure that a horizontal, non-rotating control is always available. The cells are placed inside the small hollow fiber inside the rotating chamber, the ends are sealed with wax, and the fiber is held taut by the spring loaded mount. This ensures that the fiber is always held at precisely the center of rotation. A tachometer for monitoring the speed of rotation is outside the incubator.

Developmental changes in muscle cells. The first series of micrographs in this report were taken from control fibers (i.e., fibers that were held in the horizontal position and which were not rotated). After three days in hollow fibers, muscle cells have initiated the process of fusion and myofibrillogenesis. As shown in the electron micrographs in Figures 1 and 2, fusion has been initiated as indicated by the presence of adjacent nuclei in multinucleated myotubes. Additionally, the fact that myofibril assembly has been initiated is suggested by the filamentous material in the general vicinity of the nuclei in Figures 1 and 2. In some instances at this stage of differentiation, rudimentary banding patterns are being formed; however, they lack the highly organized pattern that is observed later in muscle development or in adult skeletal muscle (c.f., Figures 3 and 4).

By seven days in culture intact, functional, well-aligned myofibrils are present in large quantities (Figures 3 and 4). In many cases, the myotubes contain more myofibrils than shown in these two figures, and the side-to-side alignment of the myofibrils is sometimes not as well organized. The appearance of the muscle cells in these two figures is also representative of that which would be observed in muscle cells in culture on polystyrene culture dishes at approximately the same age.

By fourteen days in culture (Figures 5 and 6), several additional changes have occurred in the cells in hollow fibers that are also consistent with those observed in conventional cell cultures. One of the unfortunate consequences of growing skeletal muscle cells in culture in the absence on anchoring connective tissue is that the cells will sometimes contract so vigorously that they detach themselves from the surface and die (Once the cell membrane has been ruptured, the high concentration of calcium ions in the culture medium causes the fractured myotubes to contract to the point of complete disruption of the cell). Thus, after two weeks in culture many of the remaining cells are those in which no myofibrillar structures are present. Moreover, the percentage of nonmuscle cells will obviously be higher if some of the myotubes have become lost in the medium. These observations are borne out by the electron micrographs of fourteen-day cultures shown in Figures 5 and 6. Thus, for the purposes of this project, the optimum period of time to examine myofibrillar assembly is approximately one week after establishment of the cultures in the hollow fiber, and all subsequent comparisons were made at this time.

The experiment from which the electron micrographs in Figures 1 through 6 were taken was a control experiment in which the cells were held in a horizontal position and were not rotated. This is emphasized for two reasons. First, all subsequent electron micrographs of clinostat-treated samples after seven days in culture should be compared to the seven-day control samples in Figure 3 and Figure 4. Second, the developmental changes in myogenesis in the clinostat were comparable to those observed in the non-rotated control samples, with exceptions noted later in this section. In general, cells grew at the same rate, fused at the same general time and accumulated myofibrils to the same extent in both horizontally rotated samples and vertically rotated muscle samples.

Electron micrographs from muscle cells grown for seven days under conditions of horizontal rotation in the clinostat are shown in Figures 7 and 8, and micrographs from muscle grown for seven days

under conditions of vertical rotation in the clinostat are shown in Figures 9 and 10. Two general conclusions are apparent from these photographs. First, the intracellular organization in horizontally and vertically rotated samples is similar. Cross-striations of various degrees of organization are apparent in both horizontal and vertical samples, ranging from barely distinguishable banding patterns (Figure 7) to quite highly organized patterns in other cells (Figure 10). The second conclusion from this study is that the myofibrils seem generally to be more highly organized and in tighter register in the control cultures (i.e., horizontal, non-rotated) than in any of the Clinostat samples. Comparison of Figures 3 and 4 with Figures 7-10 illustrate this point. Thus, it appears that rotation of muscle samples alone has more effect on myofibrillar organization than whether the samples are rotated in the horizontal position (i.e., simulating microgravity) or in the vertical position (i.e., with the gravity vector constant on the cells).

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Figure 1. Control Muscle Cells After 3 Days of Incubation. x5,000

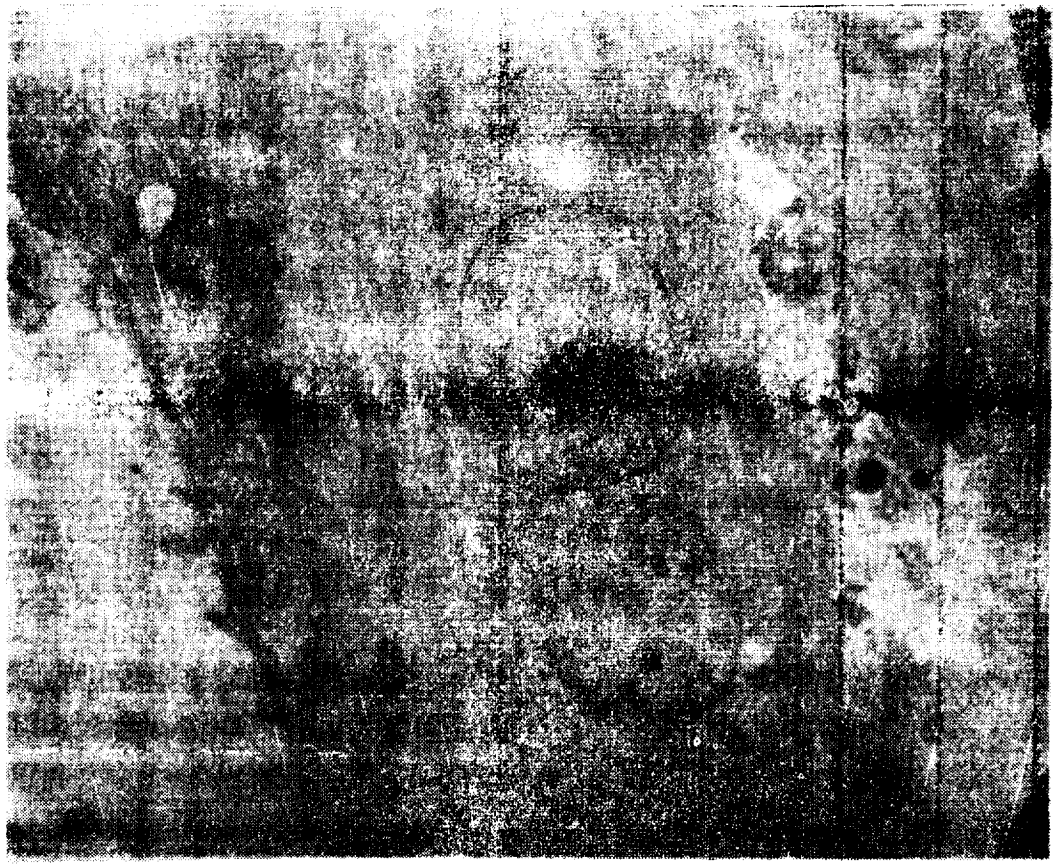

Figure 2. Control Muscle Cells After 3 Days of Incubation. x5,000

Figure 3. Control Muscle Cell After 7 Days of Incubation. x5,000

Figure 4. Control Muscle Cells After 7 Days of Incubation. x5,000

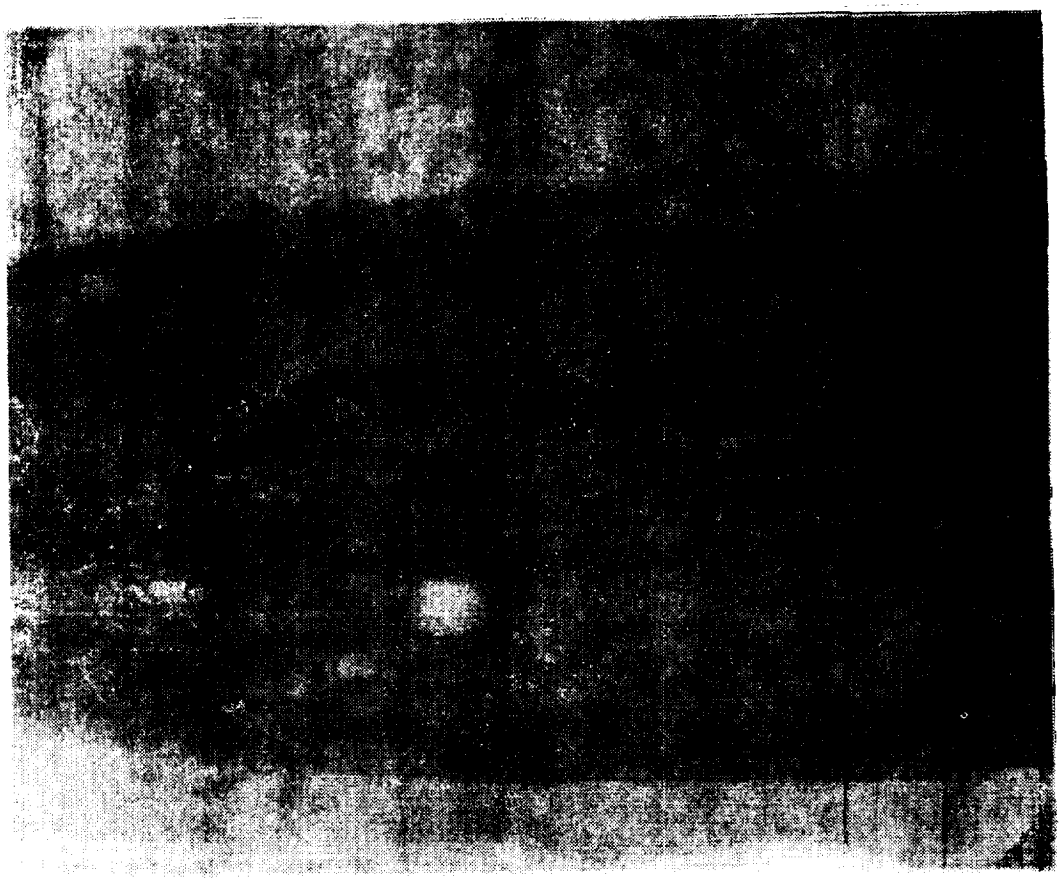

Figure 5. Control Muscle Cell After 14 Days of Incubation. x5,000

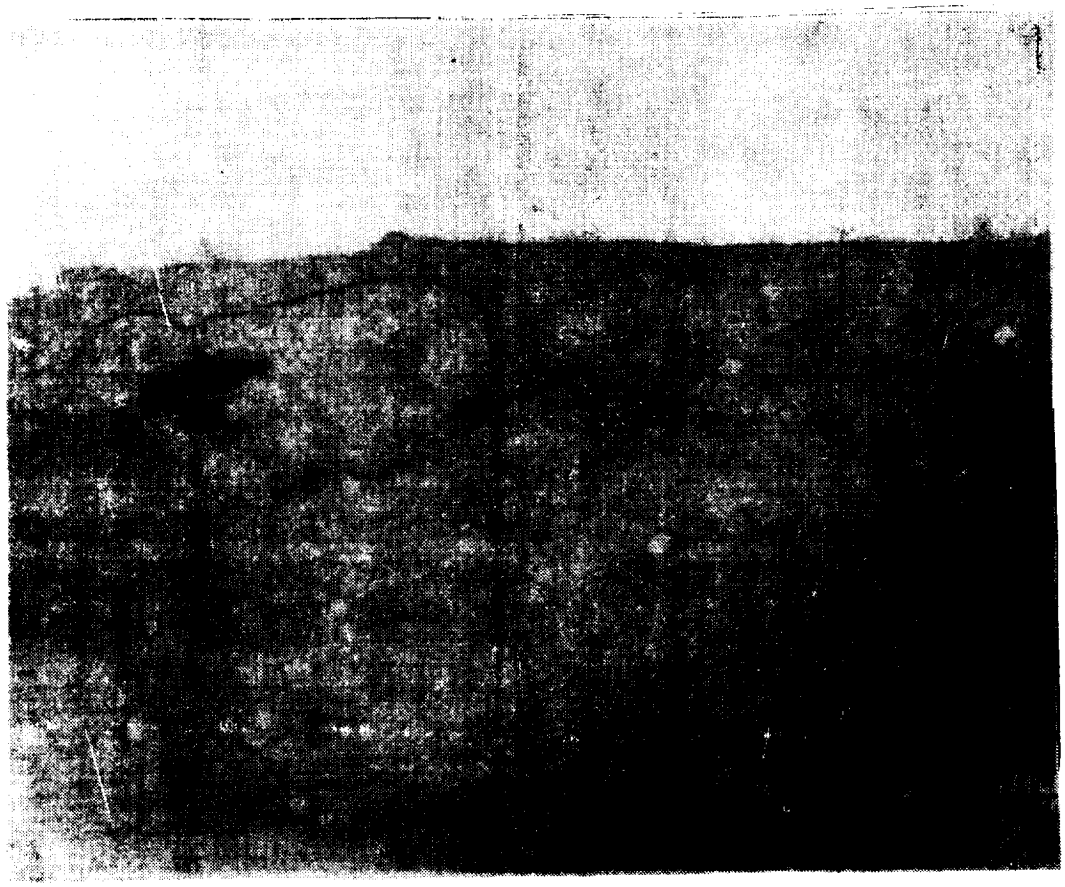

Figure 6. Control Muscle Cell After 14 Days of Incubation. x5,000

Figure 7. Muscle Cell After 7 Days of Rotation in the Horizontal Position in the Clinostat at 100 rpm. x5,000

Figure 8. Muscle Cell After 7 Days of Rotation in the Horizontal Position in the Clinostat at 100 rpm. x5,000

Figure 9. Muscle Cell After 7 Days of Rotation in the Vertical Position in the Clinostat at 100 rpm. x5,000

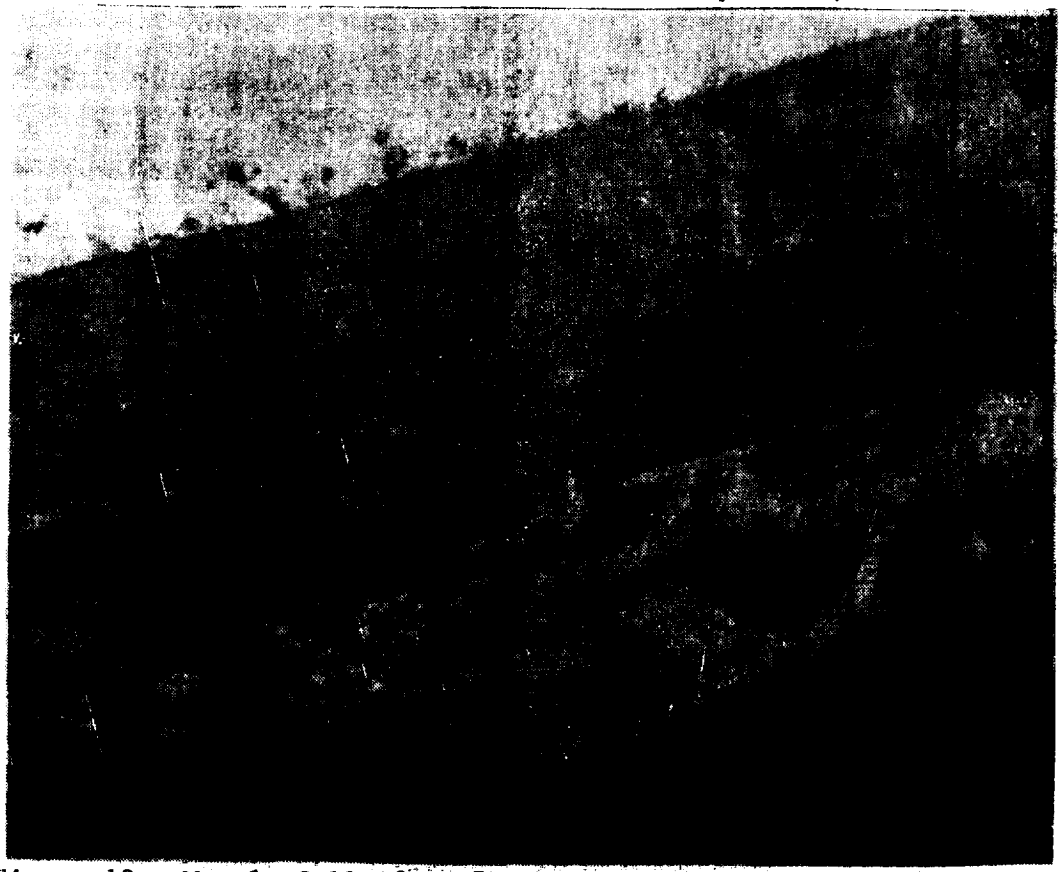

Figure 10. Muscle Cell After 7 Days of Rotation in the Vertical Position in the Clinostat at 100 rpm. x5,000